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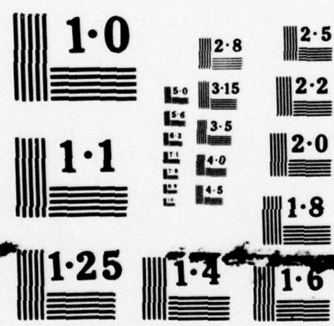

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REPORT DOCUMENTATION PAGE

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1. REPORT NUMBER: AFOSR-TR-79-0694
2. GOVT ACCESSION NO.:
3. RECIPIENT'S CATALOG NUMBER:

4. TITLE (and Subtitle): Direct Exposure of Monolayers of Mammalian Cells to Airborne Pollutants in a Unique Culture System.

5. TYPE OF REPORT & PERIOD COVERED: INTERIM Report, 6/1/77 through 5/31/78

6. PERFORMING ORG. REPORT NUMBER:

7. AUTHOR(s): R.E. Rasmussen, T.T. Crocker, G.S. Samuelson, M.E. Witte, J.T. Taylor, R.M. Hamburg

8. CONTRACT OR GRANT NUMBER(s): AFOSR-77-3343

9. PERFORMING ORGANIZATION NAME AND ADDRESS: Department of Community & Environmental Med. and School of Engineering, Univ. of Calif., Irvine, Irvine, Calif. 92717

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: 61102F 2312A5

11. CONTROLLING OFFICE NAME AND ADDRESS: AFOSR, Bldg. 410, Bolling AFB, Washington, D.C. 20332 (NL)

12. REPORT DATE: August 1978

13. NUMBER OF PAGES: 19

14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office): Annual progress rept. no. 1, 1 Jun 77-31 May 78,

15. SECURITY CLASS. (of this report): Unclassified

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE:

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

Ronald E./Rasmussen, T. Timothy/Crocker, G. Scott/Samuelson, M. E./Witte J. T./Taylor

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

22p.

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)
Mammalian cells, Air pollution, Nitrogen dioxide, Ozone, Sulfur dioxide, Cytotoxicity, Mutation.

nitrogen dioxide

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A 6-chamber system for the exposure of mammalian cells to pollutant gases has been assembled and tested. The system allows exposure of monolayers of cells to various concentrations of NO_2 and recovery of the cells for analysis. Capability for exposure to ozone and sulfur dioxide alone or in combination is being added to the system. Studies with cell lines derived from human and rodent respiratory tissue have shown that the cells are extremely sensitive to inhibition of cell replication at low levels of NO_2 (0.15 ppm) and ozone (0.05-0.08 ppm). This effect does not seem to be

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due to the solution of the gases in the growth medium but rather due to a direct effect on the cells.

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PREFACE

This is the first annual progress report submitted under a grant from the Air Force Office of Scientific Research (Grant No. 77-3343). It describes progress in the development of a unique culture system for mammalian cells which allows direct exposure of the cells to air pollutants at realistic concentrations. This project is a collaborative effort of the Department of Community and Environmental Medicine and the School of Engineering, University of California, Irvine. Project monitor was William O. Berry, Ph.D., of the Life Sciences Directorate, AFOSR.

Principal investigator was Ronald E. Rasmussen, Ph.D., Associate Adjunct Professor, Department of Community and Environmental Medicine. Co-principal investigators were T. Timothy Crocker, M.D., Professor and Chairman, Community and Environmental Medicine, and G. Scott Samuelsen, Ph.D., Associate Professor, School of Engineering, University of California, Irvine.

SUMMARY

A six-chamber system for exposure of monolayers of mammalian respiratory cells to pollutant gases and other airborne materials has been assembled and tested. This system replaces a previous two-chamber system which was used for pilot studies. The capability now exists for exposure of cell cultures to NO_2 and ozone and combinations of these gases. Any one or grouping of the six chambers may be designated as control or gas-exposure chambers. In addition, the exposure chambers themselves have been redesigned to facilitate operation of the experiment and to reduce the equilibration time required to achieve the desired test levels. Equilibration time is now 5 minutes at a gas flow rate of 4 liter/min as compared to 20 minutes required by the original design. The cell holders have also been redesigned to facilitate more rapid manipulation of cell cultures during experiments, and thereby increase efficiency.

Studies of the effects of NO_2 (0.15 ppm) and ozone (0.05-0.08 ppm) on human and rodent cells derived from respiratory tissue have shown that these gases rapidly decrease the proliferative potential of the cells when the exposure is carried out in the special exposure system. Exposure of cells to liquid medium containing nitrite ion at high concentration did not produce similar cytotoxic effects. A tentative conclusion is that the gases interact directly with the cells rather than by first dissolving in the growth medium.

SECTION I

CELL CULTURE EXPOSURE SYSTEM

G.S. Samuelsen, J.T. Tay[redacted] R.M. Hamburg

INTRODUCTION

The two-chamber exposure system used previously consisted of an NO_2-exposure chamber and a control chamber. The system has been expanded to six chambers to provide the increased capability for:

(1) A maximum of five gas-exposure chambers
(2) Individual flow controls on each exposure chamber to allow <u>simultaneous</u> exposure to a range of concentrations
(3) Generating and monitoring atmospheres containing two pollutant gases (NO_2, O_3) and combinations of these gases.
(4) Later expansion for generating and monitoring atmospheres containing additional pollutant gases (e.g. NO_2, O_3, SO_2, organics) and combinations of these gases.

In addition, the cell culture exposure chambers and cell holders have been redesigned for use in the six-chamber exposure system.

The redesigned exposure system is shown in Figure 1. During the present grant period, the original system has been employed for the biology-effects testing in parallel with the design and construction of the new six-chamber exposure system.

The results of the biology-effects testing are described later. The materials and methods, and experimental results and discussion associated with the development and operation of the two-chamber system are described elsewhere.[1] The present report describes the design and evaluation of the six-chamber system, and redesigned cell culture exposure chambers and cell holders.

MATERIALS AND METHODS

Six-chamber Exposure System

The six-exposure chambers are presently arranged to provide 5 cell culture gas-exposure chambers and 1 cell culture control chamber. The design of the system allows any combination of control and gas-exposure chambers to be employed. An exposure temperature corresponding to that prevailing in the respiratory tract (37°C) is maintained by enclosing the chambers in temperature controlled incubators. The inlet stream to the chambers is composed of purified air containing 5% CO_2. The cell culture media requires 5% CO_2 to maintain the proper pH. Compressed house air and a cylinder of CO_2 are used as the sources for the inlet stream mixture. The compressed house air is purified by passage through a 5.0 μm filter, a Purafil filter (removal of contaminant gases NO_x, SO_x), an activated charcoal filter (removal of O_3 and organics), and a 0.4 μm bacteriological filter. The airways and chamber walls are sterilized to eliminate contaimination by airborne

micro-organisms.

The flows of air and CO_2 are metered by use of stainless steel rotameters. A flow rate of 4 liter/min is maintained to each chamber in order to provide sufficient flow to all of the monitoring instruments and acceptable air exchange within the exposure chambers. Before entering the chambers, the air/CO_2 mixture is prewarmed to 37°C by passage through a coil of flame-sterilized tubing within each incubator. The mixture may also be passed through two gas washing bottles in series should elevated humidification be desired. Prior to entering the exposure chamber, the appropriate concentration of NO_2 is injected. The source of NO_2 is a cylinder of compressed gas. Precise control of NO_2 flow to the chambers is maintained by flow restrictors (porous metal structures). Changes in exposure concentrations may be accommodated with ease. Due to the high reactivity of ozone and potential loss of ozone in the chamber feed lines, each exposure chamber has its own ozone generator.

To prevent condensation of moisture, heated sample lines are used to conduct the gas stream to the Dasibi ozone monitor and Beckman NO_2-to-NO convertor both of which are maintained in a constant temperature oven (38°C). Solenoid valves located in the oven enable alternate sampling of the individual chambers. The solenoid valves are controlled by a gas flow sequencer which provides independent manual and automatic sequential control. The sequencer has two modes of operation to allow flexibility in monitoring and controlling gas flow from the exposure chambers. The manual mode allows individual on-off control of any valve for pre-experimental set up. The automatic mode steps the sequencer automatically through a predetermined sequence enabling gas flow between selected chambers and a common entry to the instruments used for monitoring. Thermocouples are located in the incubator vent line to insure the heated sample lines are maintained at the correct temperature.

Continuous monitoring of the chamber atmosphere is necessary to insure that deviation from the set exposure conditions does not occur. Relative humidity is monitored continuously using a Brady Array Model SC1021-M. NO or NO_x is monitored continuously using a Beckman 952 chemiluminescent analyzer. The gas stream flows through a heated vent line either to the instrument directly or to the external NO_2-to-NO Converter located in the 38°C oven. After conversion the NO (which is relatively insoluble in water) is passed through a water trap. The dry gas is then fed to the chemiluminescent analyzer and analyzed for the NO and NO_x concentration. Ozone is monitored continuously by the Dasibi ozone monitor model 1003AH. An Esterline Angus multipoint recorder provides a permanent record of the NO_2 concentration (as NO) during the course of an experiment.

The system has been designed to allow the later addition of an SO_2 monitor.

Cell Culture Exposure Chamber

These cell culture exposure chambers have been redesigned to reduce physical size, to make it easier to install the cell holders, and to facilitate conduct of the exposure tests.

The chambers which were previously used had a volume of approx 0.9 cu. ft. while the new chamber (which is a 4 in x 4 in x 14 in. rectangle) has a vol. of

approx 0.1 cu ft. One side of the chamber is a silicone gasketed door which allows free access to all parts of the chamber. A baffle across the inlet end of the chamber assures a more laminar gas flow throughout. Each cell holder is held tightly in place by a fixture designed for that purpose. After inserting the cell holders, the medium feed lines and withdrawal lines can be easily attached. When the installation within the chamber is complete, the entire exposure chamber is suspended in the incubator at a front to back angle of about 45°. This assures that the medium covering the cells will be minimal and can be drained off through the withdrawal system.

The material used to fabricate the new chambers is Lexan, the same material as used in the previous chambers.

Cell Holders

The cell holders previously used required great care and dexterity to assemble. In addition, the holders, while in use, were inverted and the effluent medium dripped down into collection dishes. This method was adequate unless there was a need to analyze the medium. After a four hour exposure, medium collected in this manner is too dehydrated to be analyzed. The method also proved to be messy.

The redesigned cell holders, shown in Figure 2, are simplified with respect to assembly and now incorporate the features necessary for vacuum withdrawal of the medium. The new system for medium removal incorporates a suction line (using a few inches of water vacuum) which deposits the medium from the cell holders in individual vials. The withdrawal tubes are made of sterilizeable TFE Teflon since they contact the cell holders in the area where the cells are located.

Experimental Results and Discussion

This section describes the tests undertaken or planned to evaluate the performance of the six-chamber exposure system, the cell culture exposure chambers, and the cell holders.

Six-chamber Exposure System

The evaluation of the six-chamber exposure system consisted of five bench tests (to assess the performance of system components prior to fabrication of the system) and a test for the completed system. Each of the tests are summarized below.

Stability of Various Ozone Generators

Tests were conducted to assess the performance of various ozone generators in the range of 0.05 ppm to 1.00 ppm at a 4 l/min flow. All but one generator were found to be unstable and not suitable for consistent low level generation as required by the exposure system. The Dasibi generator, marketed as a calibrator for the ozone monitor, was determined to meet the performance criteria.

The Dasibi O_3 generator was stable at all settings and at the 37°C working temperature (See Figure 1). The average fluctuation from the mean generated O_3 concentration was 3%.

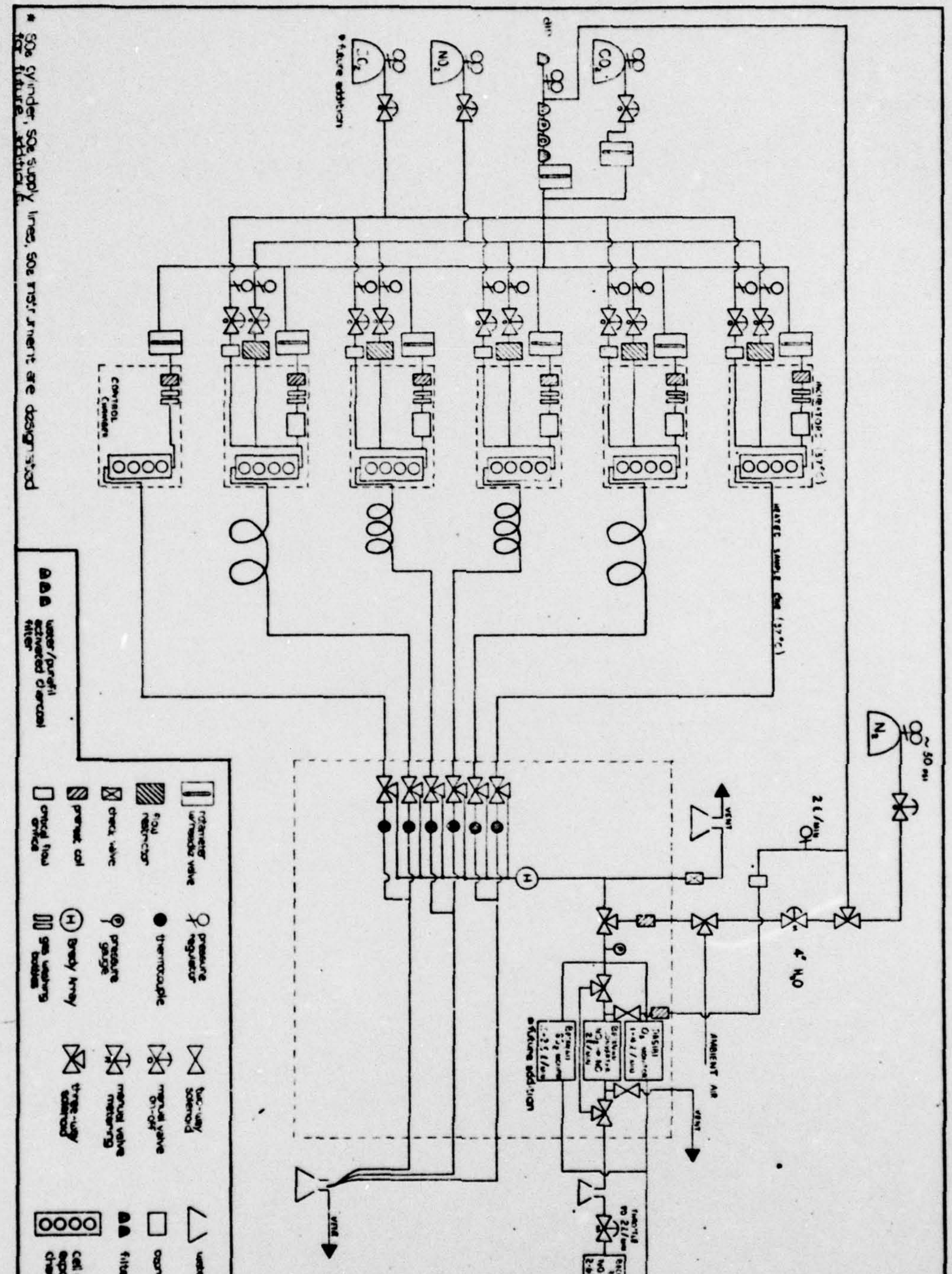

Figure 1. Six-chamber Exposure System

CELL HOLDER ASSEMBLY

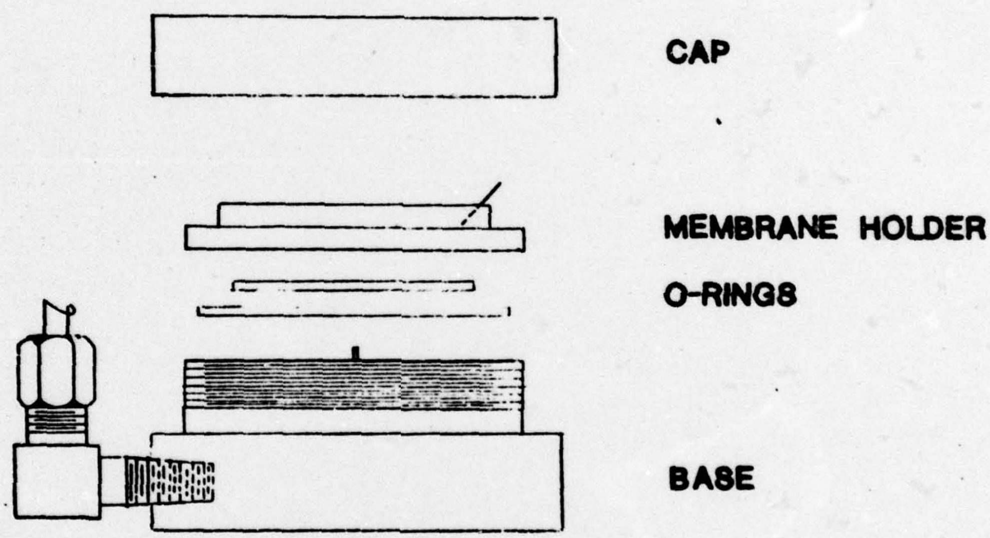

Figure 2. Cell Holder Assembly

O_3 and NO_2 Interferences

Bench tests were conducted to identify potential interferences in the simultaneous generation and monitoring of ozone and nitrogen dioxide. Tese results yielded no evidence of interference effects in either nitrogen dioxide monitoring or ozone monitoring for ozone concentrations less than 1.0 ppm and NO_x concentrations less than 0.5 ppm. Interference effects were evident for high concentrations (i.e. 5.0 ppm) of NO_2. In the presence of 5.0 ppm NO_2, the Dasibi O_3 monitor displayed up to 14% less O_3 at 0.27 ppm O_3, and 10.5% less O_3 at 0.16 ppm O_3. No effect was observed when monitoring 0.07 ppm O_3. These interferences do not pose an immediate threat since the exposure concentrations are not presently anticipated to reach levels that produce interferences.

Quality of Inlet Air

The two-chamber system employed a Purafil column to remove NO and NO_2 from the inlet air. Tests were undertaken to monitor the quality of inlet air over prolonged time periods and determine whether the Purafil alone would be suitable for the six chamber system. The monitoring data indicated that (1) the air source humidity periodically decreases below the minimum level (10% RH) required for Purafil actuation, and (2) levels of various pollutants (e.g. NO, NO_2, CO) can rise to levels of incipient concern, an effect presently attributed to fluctuations in ambient conditions. As a result, the Purafil purification column was modified to include water addition and activated charcoal for the six-chamber system. The water addition provides the necessary relative humidity to initiate the Purafil contaminant removal process. Preliminary tests of the system indicate that NO is removed entirely, and NO_2 is removed to an acceptable (0.002 ppm) level.

Sample Lines

A bench test was conducted to determine O_3 losses in Teflon line of varying lengths. Results indicated that no chemical transformations of O_3 occur in Teflon line of lengths up to 10 ft. These results are consistent with tests conducted previously for NO_2.

Additional tests are planned to assess the effect of elevated temperature (37°C) on O_3 and NO_2 losses in Teflon line.

System Evaluation

The six-chamber system has been evaluated for performance on NO_2. Flow rates of 4 l per min. were set for each of the six chambers. Various pressures of NO_2 were set on the flow restrictors of each chamber and the outputs were monitored over an eight-hour period. The performance was judged to be satisfactory and stable. Tests with ozone and combined mixtures of ozone and nitrogen dioxide are in progress.

Cell Culture Exposure Chambers

The evaluation of the exposure chamber redesign involved the bench test described below. An assessment of the chamber peformance in a cell exposure test is planned in the continuation year.

Chamber Degradation

Tests have been conducted with the redesigned chambers to identify potential problems with nitrogen dioxide and ozone degradation and/or absorption in the exposure chambers. Internal probing of the exposure chambers has demonstrated that the NO_2 concentration and O_3 concentration remain uniform throughout the chamber. This is in contrast to the larger Rochester chambers for which the concentration of NO_2 was discovered to decrease during transport through the chamber. Using glass vessels and Lexan chips, the Lexan was found to absorb, but not react with NO_2. The absorption of NO_2 was overcome by coating the interior of the exposure chambers with silicone grease which greatly reduced the NO_2 loss. The difference in performance of the redesigned chamber is not yet explained.

Cell Holder

The redesigned cell holders and media removal system have been fabricated. The system will be evaluated early in the continuation year.

SECTION II

CELL EXPOSURE STUDIES

R. E. Rasmussen, M. E. Witte, T. T. Crocker

CELL EXPOSURE STUDIES

Protocol for Cell Exposure

The response of cells to pollutant gases may be affected by their physiological state at the time of exposure. Although specific experiments have not been done to study this possibility, the methods of cell culture leading up to the exposure have been standardized so that the cells will be in a similar state of growth for each experiment.

The growth medium for the cells is Eagle's minimal essential medium prepared from the powdered form supplied by Grand Island Biological Co. and supplemented with 10% fetal calf serum and antibiotics (100 units/ml penicillin, 100/ml streptomycin, 0.25 μg/ml Fungizone). The same medium is used for perfusion of the cells during exposure.

At 24 hours before gas exposure, the cells are subcultured into 250 cm^2 plastic flasks with fresh medium at a density which will ensure that they are actively growing at the time of preparation for exposure. To prepare cells for exposure, they are released from the flask surface with trypsin (0.25%) and suspended in growth medium at a measured concentration. Aliquots of this suspension are then taken to provide known numbers of cells for planting on the Millipore filters.

The cells are impinged onto the filters with gentle suction, in a masked-off area in order to ensure that all cells on the filter will be in the area to be exposed to the gases. The cells immediately adhere to the filters, and can then be assembled into the filter holders for exposure. In the majority of the experiments, the exposure of cells to the gases was begun within an hour after planting the cells on the filters.

Cell Lines Used in Experiments

The majority of experiments were done with line V-79. This cell line was derived from Chinese hamster lung tissue, and has an indefinite life-span in culture. Is a "pseudodiploid" line with a modal chromosome number of about 22 as estimated from our cultures. The true diploid number is 22.

A human cell line (CCL-185) was used in some studies. This cell line was originally derived from a human lung carcinoma, and was obtained from the American Type Culture Collection. Based on its morphology in culture and certain metabolic characteristics, it is thought that the carcinoma may have arisen from an alveolar type 2 cell. Therefore, it provides a lung cell line of human origin for use in the gas exposure system.

Attempts to Establish Cell Strains from Rat Lung Tissue

Two cell strains have been established from tryptic digests of adult rat lung tissue. The cells have been cloned, and have modal chromosome numbers of 43 and 44 (the diploid number is 42). They are epithelial in growth habit, and resemble type 2 cells in some respects. They are presently being evaluated for possible use in the exposure system, and have not been exposed to NO_2 or O_3.

Cytotoxic Effects of NO_2

Cell Line V-79. During the present year further experiments were done to establish the response of V-79 cells to NO_2. These experiments differed from earlier ones in that the cells were planted on the filters during their active growth phase and also planted on the filters so that they would not be shielded from the atmosphere (e.g., by a meniscus of medium at the edge of the exposed area of the filter). The results of these studies confirmed previous findings of high sensitivity to inhibition of colonial growth following exposure to low levels of NO_2. Table 1 summarizes the results with V-79 at 0.15 ppm of NO_2.

Table 1

Cytotoxicity of NO_2 to Line V-79 cells exposed at a concentration of 0.15 ppm.

Hours of Exposure	Surviving Fraction
2.0	0.83 ± 0.19
3.3	0.32 ± 0.06
4.0	0.24 ± 0.08
4.0	0.[illegible]8 ± 0.09
5.0	0.08 ± 0.02
5.0	0.04 ± 0.01
6.0	0.06 ± 0.02
7.0	0.03 ± 0.02

Cell Line CLL-185. Using methods similar to those described for line V-79, cells of line CCL-185 were planted on filters and exposed to NO_2 at 0.15 ppm. The results (Table 2) showed that this cell line was also very sensitive to inhibition of colony formation by NO_2, Exposure of the individual cells to NO_2 for more than 5 hours resulted in the loss of colony-forming ability for more than 90% of the cells.

Table 2

Cytotoxicity of NO_2 to Line CCL-185 Human Lung Cells at a Concentration of 0.15 ppm.

Hours of Exposure	Surviving Fraction
2.0	0.49 ± 0.16
3.0	0.50 ± 0.35
3.0	0.25 ± 0.12
4.0	0.20 ± 0.17
4.0	0.07 ± 0.01

Table 2 (continued)

Hours of Exposure	Surviving Fraction
5.0	0.11 $\pm$ 0.07
6.0	0.04 $\pm$ 0.03
7.0	0.02 $\pm$ 0.01

In order to determine whether the inhibition of colony formation by V-79 at 0.15 ppm of NO_2 could be produced by longer exposure to a lower concentration, cells on filters were exposed to 0.07 ppm for 6 hours and the results compared with those found after exposure to 0.15 ppm for 3 hours. In the latter case, the surviving fraction was 0.49, and after 6 hours at 0.07 ppm it was 0.47, indicating that the loss of colony forming ability is the result of an accumulation of damage induced by NO_2. Experiments with lower concentrations are planned to determine whether a threshold exists for the observed cytotoxic effect.

Cytotoxicity of Ozone, O_3

A preliminary experiment has been done with cell line CCL-185 in order to estimate the range of concentrations of O_3 in which future experiments will be conducted. The results, shown in Table 3, suggest that O_3 is somewhat more toxic in this assay than is NO_2. The concentration of O_3 in this experiment ranged between 0.05 at the beginning of the exposure to a maximum of 0.08 ppm at the end. The variation was probably due in part to absorption of the gas by the exposure chamber walls and other materials in the chamber early in the exposure period. Bench tests are proceeding to minimize this problem in future experiments.

Table 3

Cytotoxic effect of O_3 on-line CCL-185 cells. 'The concentration of O_3 was 0.05 ppm at the beginning of exposure and was 0.08 ppm at the end.

Hours of Exposure	Surviving Fraction
3	0.31 $\pm$ 0.4
4	0.17 $\pm$ 0.08
5	0.28 $\pm$ 0.38
6	0.17 $\pm$ 0.19

Studies on the Mechanism of Cell Death After Exposure to NO_2

Experiments conducted prior to the beginning of the present project suggested that cells exposed to NO_2 in the exposure system were either detaching from the filters or disintegrating. This conclusion was supported by two findings. First, when cells were exposed as nearly confluent layers on the filters and then recovered and planted as individual cells to estimate survival, all cells that were recovered from the filters were subsequently able to form colonies. Second, the number of cells recovered from those filters exposed to NO_2 was always smaller than that recovered from the filters exposed to air. When cells were exposed to NO_2 and then examined microscopically after fixation and staining, it appeared that the exposed

cells were more rounded up as if they were less firmly attached to the filters.

In order to further study this effect of NO_2, we have conducted a series of experiments with cells labeled with ^{3}H-thymidine to label the nuclear DNA and ^{14}C-amino acids to label cellular protein. The data from 5 such experiments are shown in Table 4.

Table 4

Radioactivity remaining with filters carrying radiolabeled cells after exposure to air or NO_2. Line V-79 cells were prelabeled with ^{3}H-thymidine and ^{14}C-amino acids and planted on filters and exposed to air or NO_2 as described in the text. Following exposure, the filters were washed with saline, dried, and immersed in scintillation solution for counting. The values are DPM per filter, and are the mean of counts from four filters.

Experiment No.	Exposure	^{3}H-DPM x 10^{-5}	NO_2/Air	^{14}C-DPM x 10^{-3}	NO_2/Air	^{3}H/^{14}C
15	NO_2	1.7	0.23	1.2	0.32	142
	Air	7.5		3.7		203
17	NO_2	0.98	0.58	3.8	0.64	25.8
	Air	1.7		5.9		29.7
18	NO_2	0.85	0.34	2.8	0.53	30.4
	Air	2.5		5.3		47.2
21	NO_2	4.6	0.41	25.0	0.57	18.4
	Air	11.2		44.0		25.4
25	NO_2	3.6	0.68	14.0	0.78	25.7
	Air	5.3		18.0		29.4
21	Not exposed	11.0		44.0		25.0
25	Not exposed	5.5		19.0		28.9

In all experiments, the labeled cells were planted as usual on the filters in numbers that produced a confluent cell layer by the time of exposure. After exposure to 5 ppm of NO_2 for 2 hours, the filters were removed from the holders, washed with saline, air-dried, and immersed in scintillator solution containing a solubilizer. The radioactivity was determined by double-label counting in a Nuclear-Chicago scintillation spectrometer, and the proportions of the tritium and carbon labels determined using labeled toluene standards and simultaneous equations. In experiment #15, the ratio of ^{3}H-DPM/^{14}C-DPM in the labeling medium was 100:1; in all other experiments, it was 10:1.

The results in Table 4 clearly show that exposure to NO_2 affects the labeled cells in such a way that they become less firmly attached to the filters. In every experiment, both ^{3}H and ^{14}C are lost from the filters exposed to NO_2. The extent of loss was variable among the experiments for as yet undetermined causes.

There was no loss when the cells were exposed to air as indicated in experiments 21 and 25. In the latter experiments, the amounts of ^3H and ^{14}C remaining on the filters exposed to air were the same as on filters held in immersed culture for the same period.

A further interesting point is that in every experiment, the ratio of $^3H/^{14}C$ remaining with the filter was always lower in the case of the NO_2-exposed filters than the air-exposed filters. This suggests that more nuclear DNA is being lost than other protein-containing cellular components. A possible interpretation, that is consistent with other observations is that the cells accumulate a certain amount of damage at which point they break apart, releasing the cell nucleus, and leave part of the cell still attached to the filter. This is further supported by the finding of anucleate cells or "ghosts" on NO_2-exposed filters that have been fixed and stained.

A possible trivial explanation could be that NO_2 in some way modified the filter material itself so that it caused the detachment of cells. To investigate this, filters were assembled into the holders and exposed to NO_2 at 5 ppm for 6 hours. Immediately following the exposure, the filters were removed and seeded with line V-79 cells to determine their ability to support cell growth. The results showed no differences among filters exposed to NO_2, air, or not exposed in their ability to serve as a substrate for cell growth and colony formation.

Mutagenesis Studies

In preparation for mutagenesis studies with NO_2 and O_3, experiments with cell lines V-79 and CHO have been done to establish the optimum conditions for the detection of mutants in these lines. As a mutagenic agent, ethylmethane sulfonate (EMS) was chosen because it is known to be a potent mutagen, and it requires no metabolic activation but reacts directly with cellular DNA. Cultures of V-79 or CHO cells were exposed for 2 hours to EMS in serum-free medium at a concentration of 10 mM. Following the treatment, the cells were returned to complete medium, and, when confluent, subcultured. At intervals, samples were taken and planted in medium containing 6-thioguanine (6TG) at 30 ug/ml in order to select mutants resistant to 6TG. Table 5 summarizes the results of these studies which indicate that a post-treatment period of approximately 200 hours is required to enable detection of the maximum numbers of mutants. This corresponds to about 17 generation times for both lines CHO and V-79. For cell lines or strains with longer generation times, the post-treatment incubation period can be expected to be somewhat longer.

Table 5

Mutagenesis of Cell Lines V-79 and CHO by Ethylmethanesulfonate (EMS). The values are colonies formed when 10^5 surviving cells were planted in 100 mm diameter dishes and incubated with 6TG at 30 ug/ml, and are based on at least 5 replicates.

Table 5 (continued)

		Hours After Exposure to EMS								
		72	96	144	200	216	312	374	408	504
Line V-79										
Expt. #1	EMS	2	2	18±4	39±7					
	CONTROL	1	1	1	0					
Expt. #2	EMS			29±5		29±6	39±7		30±14	34±6
	CONTROL			3		2	4		4	4
Line CHO										
	EMS					50±8	67±14	56±10		53±6
	CONTROL					1	3	3		2

Experiments to test the mutagenic action of NO_2 and O_3 and their possible chemical products which may be formed upon solution or reaction with compounds in the cell growth medium are in progress. Preliminary studies have been done to estimate the possible toxicity of solution products of NO_2 and SO_2 for cell lines V-79 and CCL-185. These studies(Tables 6 and 7)consisted of exposing cell cultures to solutions of $NaNO_2$ or Na_2SO_3 in phosphate buffered saline (PBS) at neutral (pH 7.0) and acid pH (pH 4.0) and then determining cell survival in terms of the ability to form colonies. The results showed that at pH 7.0, solutions of $NaNO_2$ were not toxic at the highest concentration tested (10^{-2} M) but when exposed at pH 4.0, significant toxicity was seen at 10^{-3} M. Similar results were seen with Na_2SO_3, except that line CCL-185 was killed by NA_2SO_3 at 10^{-2} at pH 7.0. The toxic effect at pH 4.0 can be attributed to the formation of nitrous or nitric acids from $NaNO_2$ and sulfurous acid from $NaSO_3$. It has not yet been determined whether significant amounts of these acids may be formed in the cell culture medium in the present gas exposure system.

Table 6

Cytotoxicity of $NaNO_2$ to Cell Lines V-79 and CCL-185.

Cells were planted at 200 per 60 mm dish and allowed 3-4 hours for attachment. At that time the medium was replaced with PBS containing $NaNO_2$ at the concentration and pH indicated. After 2 hours, the medium was changed back to normal and incubation was continued for 6 days (V-79) or 10-12 days (CCL-185) to allow colony development.

Table 6 (continued)

Line V-79

Na_2SO_3, M	pH 7.0 No. of Colonies	pH 7.0 Surviving Fraction	pH 4.0 No of. Colonies	pH 4.0 Surviving Fraction
0	113.2±12.5	1.00	104.6±6.8	0.92
10^{-4}	118.4±10	1.05	121.4±9.1	1.07
10^{-3}	115.6±16	1.02	76.8±8.7	0.68
10^{-2}	110.8±2.3	0.98	0.0	0.0

Line CCL-185

0	177.8±14	1.00	174.6±11	0.98
10^{-4}	153.4±6.9	0.86	156.8±18	0.88
10^{-3}	166.8±21	0.94	8.0±6.5	0.045
10^{-2}	167.2±14	0.94	0.0	0.0

Table 7

Cytotoxicity of Na_2SO_3 to Cell Lines V-79 and CCL-185

Cells were planted at 200 per 60 mm dish and allowed 2-3 hours for attachment. At that time the medium was replaced with PBS containing Na_2SO_3 at the indicated concentrations and pH. After 2 hours, the PBS was replaced with normal medium, and incubation continued for 6 days (V-79) or 10-12 days (CCL-185) to permit colony development.

Na_2SO_3, M	pH 7.0 No. of Colonies	pH 7.0 Surviving Fraction	pH 4.0 No. of Colonies	pH 4.0 Surviving Fraction
Line-V-79				
0	82.4±6.6	1.00	90±7.6	1.09
10^{-4}	81.8±4.8	0.99	27.4±12	0.33
10^{-3}	83.6±5.8	1.01	2.6±1.8	0.032
10^{-2}	81.2±14	0.99	0.0	0.0
Line CCL-185				
0	54±6.9	1.00	51.8+9.8	0.96
10^{-4}	41.6±6.5	0.77	51.6±6.8	0.96
10^{-3}	43.2±9.9	0.80	8.6±4.5	0.16
10^{-2}	0.0	0.0	0.0	0.0

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